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Title: Estimating the Transfer Range of Plasmids Encoding Antimicrobial Resistance in a Wastewater Treatment Plant Microbial Community

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25 **Abstract**

26 Wastewater treatment plants (WWTPs) have long been suggested as reservoirs and sources of
27 antibiotic resistance genes (ARGs) in the environment. In a WWTP ecosystem, human enteric and
28 environmental bacteria are mixed and exposed to pharmaceutical residues, potentially favoring
29 genetic exchange and thus ARG transmission. However, the contribution of microbial communities
30 in WWTP to ARG dissemination remains poorly understood. Here, we examined for the first time
31 plasmid permissiveness of an activated sludge microbial community, by utilizing an established
32 fluorescent bioreporter system. The activated sludge microbial community was challenged in
33 standardized filter matings with one of the three multi-drug resistance plasmids (pKJK5, pB10 and
34 RP4) harbored by *Escherichia coli* or *Pseudomonas putida*. Different donor-plasmid combinations
35 had distinct transfer frequencies, ranging from 3 to 50 conjugation events per 100,000 cells of the
36 WWTP microbial community. In addition, transfer was observed to a broad phylogenetic range of
37 13 bacterial phyla with several taxa containing potentially pathogenic species. Preferential transfer
38 to taxa belonging to the predicted evolutionary host range of the plasmids was not observed.
39 Overall, the ARG dissemination potential uncovered in WWTP communities calls for a thorough
40 risk assessment of ARG transmission across the wastewater system, before identifying possible
41 mitigation strategies.

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49 **Introduction**

50 Wastewater treatment plants (WWTPs), at the interface between hospital/residential sewage and
51 recipient surface water, have been proposed as overlooked reservoirs of antibiotic resistance genes
52 (ARGs).¹⁻³ Indeed, there, the microbiomes indigenous to WWTP are intensely mixed with
53 microbiomes of human enteric origin, in the presence of pharmaceutical residues and other selective
54 agents, potentially stimulating the transfer of ARGs from pathogens and commensals to
55 environmental bacteria. Among the gene transfer processes (e.g., transformation, transduction and
56 conjugation), plasmid-mediated conjugation is characterized by its efficiency, even across distantly
57 related taxa for broad host range plasmids. Therefore, the transfer of ARGs is facilitated by their
58 frequent location on plasmids.⁴⁻⁶ Several studies have provided evidence that WWTP microbiomes
59 can contain significant amount of plasmids encoding multi-drug resistance.⁷⁻⁹ Environmental
60 bacteria receiving these plasmid-borne ARGs can persist in the receiving environments, facilitating
61 their dissemination.^{10,11} Considering the global public health threat posed by antimicrobial
62 resistance and the obvious load from human waste collected and transported through sewage, it is
63 crucial to evaluate the potential contribution of WWTP to plasmid mediated ARG dissemination.

64
65 In order to understand the fate of ARG-carrying plasmids in WWTP ecosystems, it is necessary to
66 disentangle the roles of plasmid type, donor strain, and resident microbial community in shaping the
67 plasmid transfer host ranges. The plasmid permissiveness assay, as originally introduced by
68 Musovic et al,¹² provides a suitable platform to address this question. Combining a fluorescent
69 reporter based plasmid detection assay with fluorescence-activated cell sorting (FACS) and 16S
70 rRNA gene amplicon sequencing of transconjugant cells, it enables quantification and identification
71 of the community fraction that receives the tested plasmid upon challenging this community with a
72 plasmid donor strain.¹³⁻¹⁵ Using this approach, extremely broad transfer host ranges of IncP-1

73 conjugative plasmid pKJK5 have been detected in microbial communities from agricultural soil,^{13,14}
74 as well as from the inlet and outlet of WWTPs.¹⁵ Yet, the permissiveness of WWTP microbial
75 communities for typical and relevant IncP-1 plasmids of different subgroups has not been examined.
76 It has been argued - mainly based on metagenomic observations - that the high species diversity and
77 cellular density of WWTP microbial communities creates a locale favoring horizontal gene
78 transfer.^{8,16} Predicting the range of plasmid-mediated genetic exchange at the community level has
79 so far not been possible; host ranges inferred from bioinformatic analyses or traditional assays have
80 been skewed toward only identifying evolutionary host taxa with preexisting genomic homogeneity
81 or examining a limited number of well-studied model strains.¹⁷⁻²⁰ We believe that direct
82 confirmation and quantification of this exchange is, however, necessary and possible via plasmid
83 permissiveness assays.¹³⁻¹⁵ By quantifying and identifying the permissive fraction, one can evaluate
84 plasmid transfer potential as an essential community property and examine abiotic/biotic factors
85 (e.g., environmental conditions, plasmid/donor type and recipient community) that shape
86 permissiveness profiles, which together will help understand plasmid-mediated ARG spread.

87
88 Here, we report on the first permissiveness estimates of a WWTP microbial community towards
89 several typical conjugative plasmids, and the first exploration of association between plasmid
90 transfer and evolutionary host ranges. A WWTP community was challenged with three ARG-
91 carrying plasmids from different subgroups in the incompatibility group IncP-1 (pKJK5, pB10 and
92 RP4)¹⁷ using either the prototypic member of Enterobacteriaceae - *Escherichia coli* or typical
93 environmental bacterium - *Pseudomonas putida* as donor strains. Distinct transfer potentials were
94 observed with the highest realized in *E. coli* (pKJK5) (50 conjugation events per 100,000 recipient
95 cells). The transfer host ranges covered 13 phyla across the different donor-plasmid combinations;
96 but no preferred transfer was observed to taxa predicted to belonging to the evolutionary host range

97 of the plasmids. It is noteworthy that plasmid acquisition was observed in several taxa with
98 potentially pathogenic species. Overall, the wide transfer potential of plasmids experimentally
99 revealed in this study confirms the importance of WWTP as a unique locale for plasmid mediated
100 ARGs exchange between enteric and environmental bacteria.

101

102 **Material and methods**

103 **Donor strain and WWTP recipient community**

104 *E. coli* MG1655 and *P. putida* KT2440 (both chromosomally tagged by *lacI^q-Plpp-mCherry*)
105 carrying one of the three plasmids pKJK5 (IncP-1 ϵ), pB10 (IncP-1 β) and RP4 (IncP-1 α) (tagged
106 with *Plac-gfp*), were used as donors (each combination group will be referred to as *donor* (plasmid),
107 e.g., *E. coli* (pKJK5)) (Table S1). The donor strains were grown overnight in LB prepared as
108 described previously.^{13,14} Recipient community was phase-isolated activated sludge from a
109 municipal WWTP (Mølleåværket, Lyngby-Taarbæk, DK). Briefly, bacteria were recovered by
110 washing, sonication and settling. Cell numbers were adjusted to approx. 3.0×10^7 cells per ml for
111 filter mating assays.

112

113 **Solid surface filter mating assay**

114 Cell suspensions of donor strain and WWTP recipient community were mixed at 1:1 cell ratio and
115 immediately filtered onto 0.2 μ m Cyclopore membranes.²¹ Filters were placed on a agar-solidified
116 synthetic wastewater medium.²² After incubation (48 hours at 25°C) and GFP maturation (48 hours
117 at 4°C), transfer events were detected by epifluorescence microscopy and transfer frequency was
118 quantified as the ratio of conjugation events (GFP-positive cells or microcolonies) to the original
119 WWTP recipient cell number (CE/R), as per established procedures.^{13,14,23}

120

121 **Sorting and sequencing**

122 For each mating condition, cells from triplicate filters were combined in 0.9% NaCl solution and
123 detached by vortexing. Transconjugants and recipients were sorted using FACS by adjusting gating
124 of bacterial size (forward scatter), green fluorescence, and red fluorescence as described earlier.^{13,14}
125 Sorted cells were subject to DNA extraction using GenePurgeDirectTM agent (NimaGen, NL). 16S
126 rRNA gene fragments were amplified by the primer set 341F and 806R,¹⁵ and subjected to paired-
127 end sequencing on Illumina MiSeq platform.

129 **Sequence analysis**

130 The forward reads of the 16S rRNA gene amplicon sequencing were analyzed using the DADA2
131 pipeline to infer exact sequence variants (ESV) (Table S2).^{24,25} As estimating ESV-specific
132 permissiveness is complicated by the (potential) growth of both transconjugants and recipients
133 during mating incubation, we calculate apparent permissiveness (AP). It is defined as the ratio of
134 the relative abundance of an ESV in the transconjugant pool and in the corresponding recipient
135 community.¹⁴ AP thus accounts for the fact that the abundance for an ESV in the transconjugant
136 pool is partly dependent on their abundance in the recipient community. Phylogenetic relatedness
137 between donor and transconjugant was calculated by DistanceMatrix in R package DECIPHER²⁶
138 and its correlation with AP values was calculated with Spearman correlation coefficient.
139 Phylogenetic conservation of AP values was analyzed by calculating their phylogenetic signal in
140 corresponding ESVs by multiPhylosignal in R package picante.²⁷ Plasmid transfer host range and
141 evolutionary host range (i.e., hosts that have carried the plasmid during evolutionary time long
142 enough to leave detectable sequence traits), were compared based on previous genomic analysis.¹⁷
143 Occurrence of these evolutionary hosts in transconjugant pools was evaluated by t-test of both
144 relative abundance and AP value. All sequences were deposited in NCBI under SRA accession

number SRP133153. Method details including experimental setups and statistical analyses are provided in Supporting Information.

Results and Discussion

Transfer frequencies across donor-plasmid combinations

Transfer frequencies in the WWTP microbial community ranged from 3.39×10^{-5} to 5.05×10^{-4} CE/R (i.e., from 3 to 50 conjugation events per 100,000 recipient cells) across donor-plasmid combinations (Figure 1); comparable transfer frequencies have been measured in soil microbial communities (6.8×10^{-5} CE/R of *E. coli* (pKJK5) and 1.0×10^{-4} CE/R of *P. putida* (RP4))^{13,28} All three plasmids transferred at higher frequency from *E. coli* compared to *P. putida* with the highest transfer frequency observed with *E. coli* (pKJK5). Comparison among transfer frequencies of the three plasmids carried by the same host showed that pKJK5>pB10>RP4 in *E. coli* and pKJK5>pB10≈RP4 in *P. putida*. Despite belonging to the same incompatibility group (IncP-1), the three plasmids present genetic divergence in their transfer and regulatory regions,^{17,29} which might explain the difference in observed conjugation behavior.

Transfer host ranges for different donor-plasmid combinations

Recipient communities were distinct from transconjugant pools (NMDS, ANOSIM P-value<0.01). While post filter-mating recipient pools were distinct from the raw AS communities, reasonable diversity was retained: the Shannon diversity index decreased slightly from 5.3 to 4.7. And notwithstanding the presence of a shared core (see below) the four transconjugant pools were distinct from each other (Figure 2). As expected, recipient pools were more diverse than transconjugant pools (Shannon diversity = 4.2-4.6; unique ESVs = 229-360 vs Shannon diversity = 1.3-3.2; unique ESVs = 73-126). Interestingly, distances within transconjugant pools and recipient

169 communities of *E. coli*/*P. putida* (pKJK5) were similar (Bray-Curtis distance within transconjugant
170 pools vs within recipient pools = 0.55 vs 0.63). However, the three transconjugant pools of *E. coli*
171 (pKJK5/pB10/RP4) were clearly distinct from each other, even though their recipient communities
172 were close (Bray-Curtis distance = 0.43-0.57 vs 0.23-0.26). Hence, a plasmid type might shape
173 transconjugant pool composition more than a plasmid donor.

174

175 The transconjugants across all donor-plasmid combinations comprised 308 distinct ESVs
176 distributed over 13 phyla (Figure 2; Figure 3). While all transconjugant pools were dominated by
177 genera from the Gammaproteobacteria class including *Escherichia/Shigella*, *Pseudomonas* and
178 *Acinetobacter*, a few other Gram-negative (*Chloroflexi*, *Acidobacteria* and *Bacteroidetes*) and
179 Gram-positive (*Actinobacteria* and *Firmicutes*) taxa were also noted. Overall, plasmid transfer was
180 observed in 34-59% of the families present in the recipient community. Thirteen permissive genera
181 were shared across all donor-plasmid combinations, representing >80% of each transconjugant pool.
182 These core permissive taxa were mainly composed by *Enterobacteriaceae* and *Pseudomonadaceae*.
183 These two lineages were also detected in transconjugant pools when permissiveness of inlet and
184 outlet of the same WWTP was examined,¹⁵ indicating their possible transmission from sewage to
185 the environment. The frequent occurrence of *Acinetobacter*, *Aeromonas* and *Streptococcus* in the
186 transconjugant pools highlights the possibility of ARG transmission to (opportunistic) pathogens.
187 The high frequency and broad range of plasmid transfer to the examined WWTP community under
188 the standardized experimental conditions, in the absence of selective pressure, suggests significant
189 ability of ARG spread under actual WWTP conditions of intense microbial interaction³⁰ and the
190 presence of residual antibiotics and other relevant co-selective stressors.³¹

191

192 **Heterogeneous apparent permissiveness profiles**

193 The relative abundance profile of community members in transconjugant pools did not agree with
194 their abundance in the recipient pools, indicating that capability in receiving plasmids varied among
195 taxa (Figure S1): a few taxa with low abundance in the recipient communities were highly enriched
196 in the transconjugant pools across all donor-plasmid combinations (e.g., *Escherichia/Shigella* <1%
197 in recipient pools and >40% in all transconjugant pools; *Shimwellia* was <0.1% in recipient pools
198 but >2% in transconjugant pools with both *E. coli* (pKJK5) and *P. putida* (pKJK5) groups). On the
199 contrary, some highly abundant taxa were poorly represented in the transconjugant pools indicating
200 their poor permissiveness (e.g., *Acinetobacteria* >40% in recipient but <0.1% in the transconjugant
201 pool with *E. coli* (pB10) group). In several abundant taxa, no plasmid transfer was detected (e.g.,
202 *Flavobacterium* at 8-10% in recipient communities while absent in transconjugant pool of *E. coli*
203 (pB10)).

204

205 Phylogenetic relatedness between recipient and donor did not explain the composition of the
206 transconjugant pools for the three examined IncP-1 plasmids (Figure S1 and S2). Certainly, high
207 intra-generic transfer was observed: from donor *E. coli* to *Escherichia/Shigella* (AP up to 704.1)
208 and from donor *P. putida* to *Pseudomonas* (AP up to 294.2). However, transfer to distant
209 phylogenetic groups, even across phylum borders, was equally observed, e.g., *E. coli* (pKJK5)
210 transferred at high frequency to *Pseudobacteroides* (*Firmicutes*) (AP up to 448.1) and *Gardnerella*
211 (*Actinobacteria*) (AP up to 429.6). Hence, the AP profile did not correlate with the phylogenetic
212 distance between recipient and donor (Spearman correlation, P-value = 0.10~0.93). Within a single
213 permissive genus, APs could be similar in magnitude or vary greatly: e.g., with *E. coli* (pB10) and
214 *E. coli* (RP4), APs of *Staphylococcus* ESVs were within one order of magnitude; with *E. coli*
215 (pKJK5), APs of *Acinetobacter* and *Pseudomonas* ESVs each ranged over three orders of
216 magnitude. Such varying response at the ESV level indicates that AP is not significantly

217 phylogenetically conserved (phylogenetic signal, P-value = 0.61~0.98). Therefore, for the three
218 IncP-1 plasmids, extrapolating permissiveness of a bacterial group to other phylogenetically similar
219 groups in the WWTP community would not be valid. Future studies, including more plasmid
220 groups, and especially plasmids with assumed narrow-host-range groups (e.g., IncF and IncI), will
221 reveal the generality of this conclusion.

222

223 **Comparing transfer host range to predicted evolutionary host range**

224 While plasmid transfer host range can be inferred from experimental permissiveness assays, it is not
225 clear how this range relates with a plasmid's long-term maintenance as plasmid acquisition is only
226 the very first step of a possible long-term plasmid-host association. Since long-term adaptation
227 between plasmid and host is achieved through genomic homogenization and subsequent cost
228 amelioration, a plasmid's evolutionary host range can be inferred from genomic comparisons
229 between bacterial chromosomes and plasmids (backbone).^{17,18} For example, for the three examined
230 plasmids, pKJK5 was predicted to have been evolutionarily present in the genera *Bordetella*,
231 *Dechloromonas* and *Pseudomonas*, pB10 in *Ralstonia* and *Variovorax*, and RP4 in *Ralstonia*,
232 *Slackia* and *Pseudomonas*.¹⁷ These predicted evolutionary hosts might have more potential in taking
233 up the plasmid and expressing its genes because of the preexisting genomic homogeneity. However,
234 we did not detect such enrichment for members of the predicted evolutionary host range of the three
235 plasmids in their corresponding transconjugant pools (Table S3). Among the six genera belonging
236 to the predicted evolutionary host range of the three plasmids, only *Pseudomonas* (0.25%~17.60%
237 with AP 0.5-294.2 across all groups) and *Dechloromonas* (0.14% with AP 2.3 in *E. coli* (pKJK5))
238 were detected in transconjugant pools. Even at higher taxonomical levels, there was little indication
239 of enrichment of evolutionary host taxa in the transconjugant pools. For example, *Burkholderiaceae*
240 (family) predicted as evolutionary host taxon of pB10, were not observed in the pB10

transconjugant pool; *Burkholderiales* (order) were observed in the pKJK5 transconjugal pools with *E. coli* as donor but below 1% with AP ranging from 0.2-262.6; Gram-positive *Actinobacteria* (class) predicted evolutionary hosts of RP4, were minor fractions of the RP4 transconjugant pools (<4% with AP ranging 0.2-429.6). Hence, evolutionary host range predicted from genomic analysis does not seem to reflect extant plasmid transfer host range in WWTP microbial communities.

In this study, the dissemination potential of ARGs in environmental communities was highlighted by the high transfer frequency (up to 50 conjugation events per 100,000 recipient cells) and the broad phylogenetic transfer range (covering 13 phyla) of the three ARG-carrying plasmids in a WWTP microbial community. Taxa belonging to a plasmid's predicted evolutionary host range do not necessarily exhibit high permissiveness. The plasmid permissiveness assay as adapted here for WWTP communities provides a quantitative assessment of a community property that is essential, but not sufficient, to describe, and ultimately predict the fate of plasmids in the environment. Indeed, the potential for plasmid uptake, as measured here, is not realized *in situ* in WWTP systems, and extrapolation to real environments will require additional experiments to identify the role of the environment, including conditions of (sub)inhibitory selective or co-selective pressure.

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Conflict of interest

The authors declare no competing financial interest.

265

266 **Supporting Information**

267 Supplementary methods of plasmid donor strain and recipient microbial community, solid surface
268 filter mating assay, sorting and sequencing (sequence analysis); supplementary figures of relative
269 abundance of genera across samples, AP profile of ESVs; supplementary tables of donor strains and
270 plasmids, information of sequences, relative abundance of predicted evolutionary taxa.

271

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387 **Figure legends**

388

389 **Figure 1.** Transfer frequencies (CE/R: the ratio of conjugation events (CE) to the original WWTP
390 recipient cell number (R)) from two donors (*E. coli* and *P. putida*) carrying one of three plasmids
391 (pKJK5, RP4 and pB10) to an activated sludge microbial community. Error bar indicates 95%
392 confidence interval of three replicates.

393

394 **Figure 2.** Diversity and phylogenetic composition of transconjugant and recipient communities. (A)
395 and (B): Shannon index and NMDS (the same color scheme was applied in the two panels; for each
396 donor-plasmid combination (circle dots), dark color indicates transconjugant pools and light color
397 (within ellipse) indicates recipient pools; triangle dots indicate WWTP microbial communities. (C)
398 and (D): phylogenetic composition at phylum level and relative abundance of phyla except Gamma-
399 and Alpha-proteobacteria in transconjugant pools. (E) and (F): top 20 abundant orders and genera in
400 the transconjugant pools.

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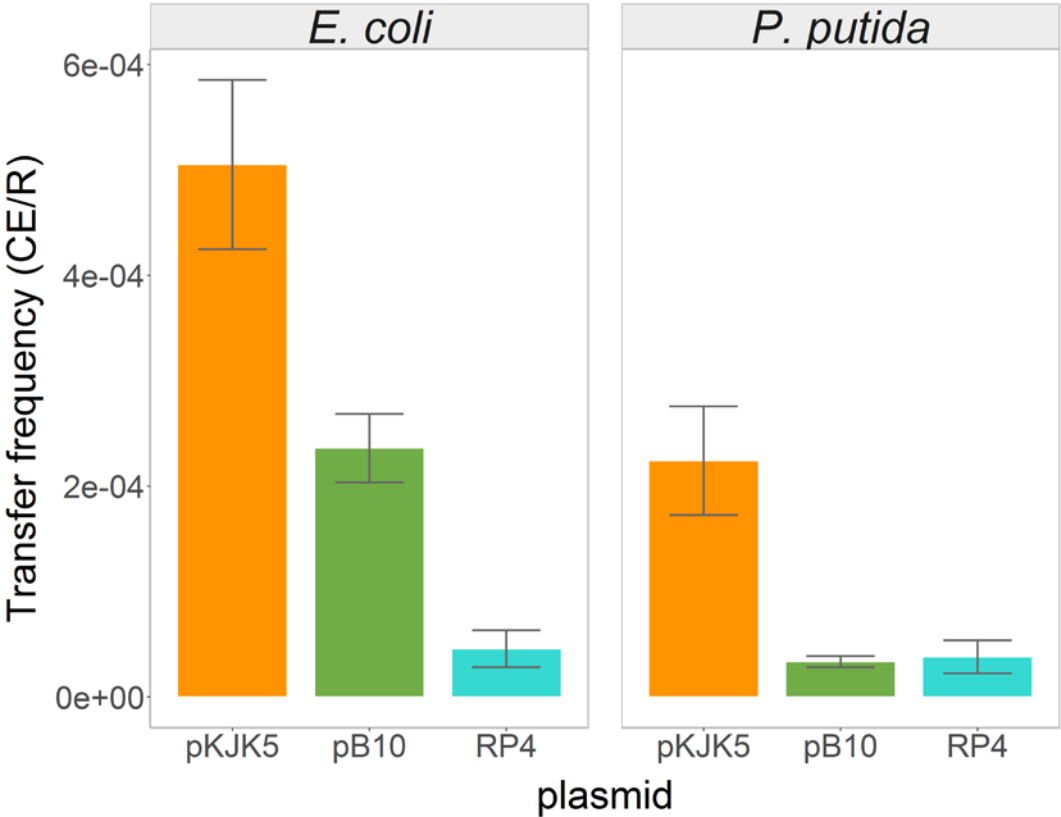
402 **Figure 3.** Composition of the transconjugant pools across four donor-plasmid combinations. (A)
403 and (B): phylogenetic tree showing the relative abundance of ESVs detected with *E. coli*
404 (pKJK5/RP4/PB10) and *E. coli/P. putida* (pKJK5) as plasmid donor. Background colors indicate
405 the 6 most abundant classes, and branch colors indicate the 13 core genera across all transconjugant
406 pools (refer to panel (E)). (C), (D) and (E): Venn diagrams at the genus level of the transconjugant
407 pools of *E. coli* (pKJK5/RP4/PB10), *E. coli/P. putida* (pKJK5) and all groups.

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410

411 **Figures**



424 **Figure 1.** Transfer frequencies (CE/R: the ratio of conjugation events (CE) to the original WWTP
425 recipient cell number (R)) from two donors (*E. coli* and *P. putida*) carrying one of three plasmids
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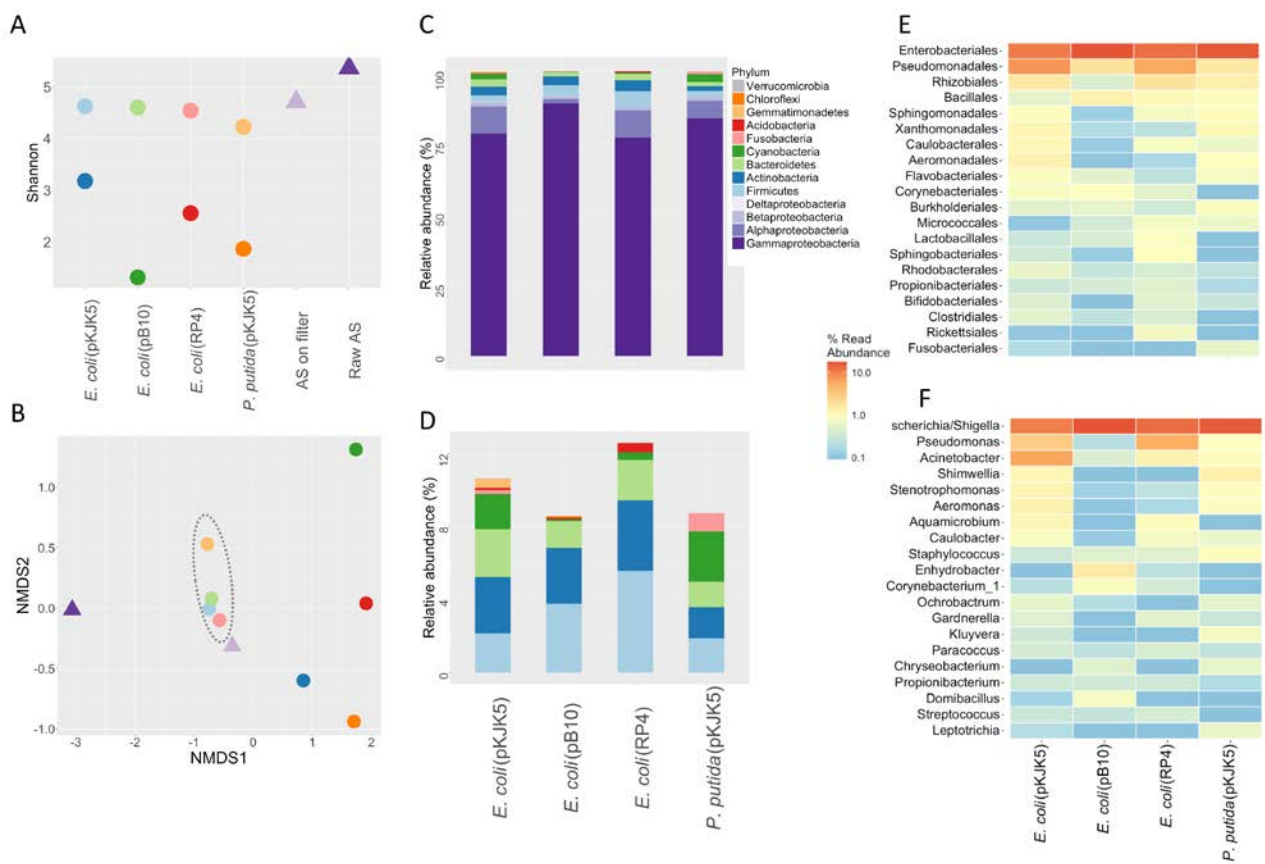


Figure 2. Diversity and phylogenetic composition of transconjugant and recipient communities. (A) and (B): Shannon index and NMDS (the same color scheme was applied in the two panels; for each donor-plasmid combination (circle dots), dark color indicates transconjugant pools and light color (within ellipse) indicates recipient pools; triangle dots indicate WWTP microbial communities. (C) and (D): phylogenetic composition at phylum level and relative abundance of phyla except Gamma- and Alpha-proteobacteria in transconjugant pools. (E) and (F): top 20 abundant orders and genera in the transconjugant pools.

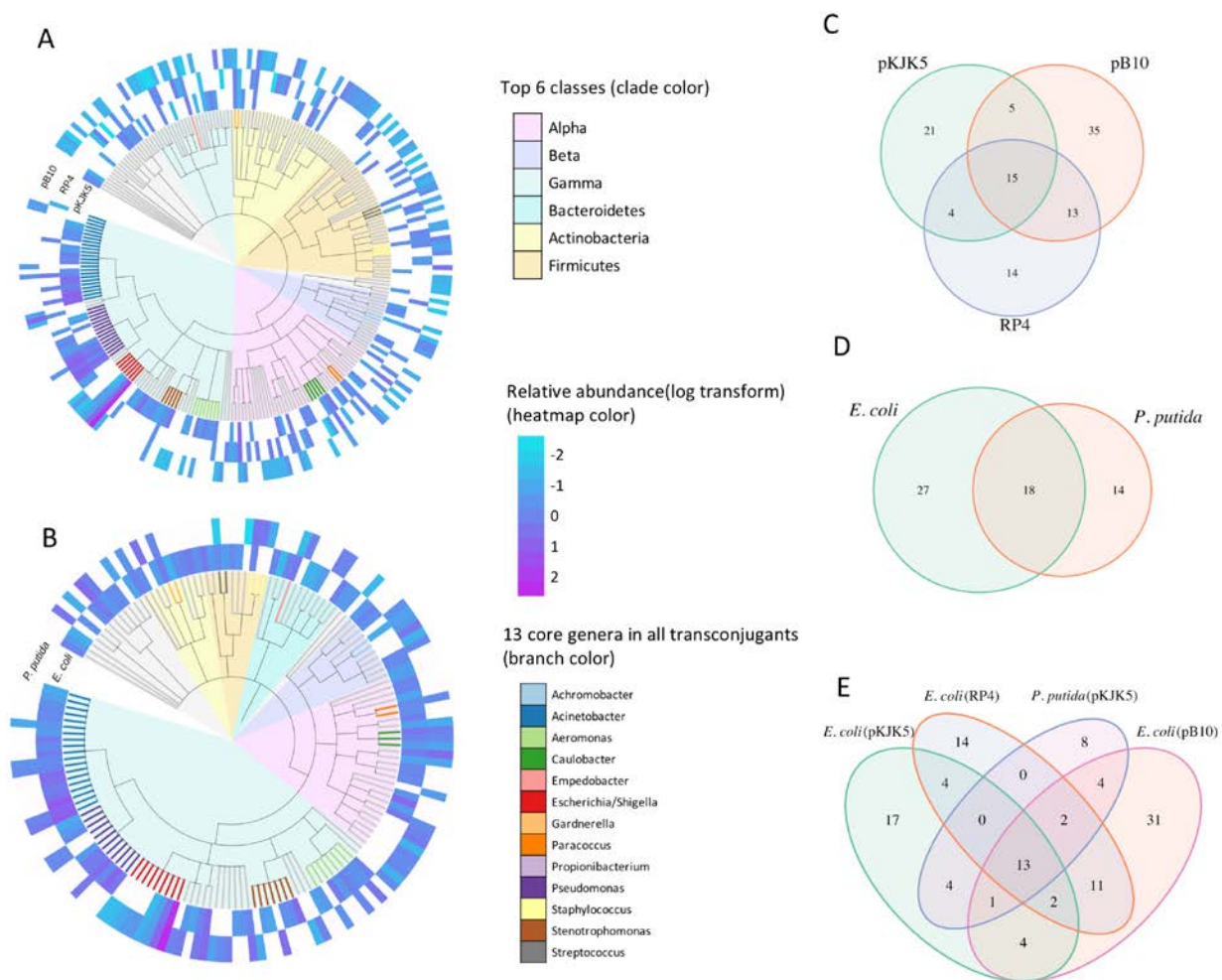


Figure 3. Composition of the transconjugant pools across four donor-plasmid combinations. (A) and (B): phylogenetic tree showing the relative abundance of ESVs detected with *E. coli* (pKJK5/RP4/PB10) and *E. coli*/*P. putida* (pKJK5) as plasmid donor. Background colors indicate the 6 most abundant classes, and branch colors indicate the 13 core genera across all transconjugant pools (refer to panel (E)). (C), (D) and (E): Venn diagrams at the genus level of the transconjugant pools of *E. coli* (pKJK5/RP4/PB10), *E. coli*/*P. putida* (pKJK5) and all groups.

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485

486 Title: Estimating the Transfer Range of Plasmids Encoding Antimicrobial Resistance in a

487 Wastewater Treatment Plant Microbial Community

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489 Liguan Li, Arnaud Dechesne, Zhiming He, Jonas Stenl kke Madsen, Joseph Nesme, S ren J.

490 S rensen, Barth F. Smets

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